Reconstituted high density lipoprotein enriched with the polyene antibiotic amphotericin B

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Abstract **The polyene antibiotic amphotericin B (AMB) is an effective antifungal agent whose therapeutic potential is limited by poor aqueous solubility and toxicity toward host tissues. Addition of apolipoprotein A-I to a multilamellar phospholipid vesicle dispersion containing 20% (w/w) AMB induces the formation of reconstituted high density lipoprotein (rHDL), with solubilization of the antibiotic. Density gradient ultracentrifugation resulted in flotation of the complexes to a density of 1.16 g/ml, and negative stain electron microscopy revealed a population of disk-shaped particles. Native gradient polyacrylamide gel electrophoresis indicated a particle diameter of ~8.5 nm. Absorbance spectroscopy provided evidence for AMB integration into the lipid milieu. AMB-rHDLs were potent inhibitors of *Saccharomyces cerevisiae* growth, yielding 90% growth inhibition at <1 μg/ml yeast culture. In studies with pathogenic fungal species, similar growth inhibition characteristics were observed. Compared with AMB-deoxycholate micelles, AMB-rHDL displayed greatly attenuated red blood cell hemolytic activity and decreased toxicity toward cultured hepatoma cells. In in vivo studies in immunocompetent mice, AMB-rHDLs were nontoxic at 10 mg/kg, and they showed efficacy in a mouse model of candidiasis at concentrations as low as 0.25 mg/kg. These results indicate that AMB-rHDLs constitute a novel formulation that effectively solubilizes the antibiotic and elicits strong in vitro and in vivo antifungal activity with no observed toxicity at therapeutic doses.**—Oda, M. N., P. L. Hargreaves, J. A. Beckstead, K. A. Redmond, R. van Antwerpen, and R. O. Ryan. **Reconstituted high density lipoprotein enriched with the polyene antibiotic amphotericin B. *J. Lipid Res.* 2006.** 47: **260–267.**

Supplementary key words apolipoprotein • phospholipid • *Saccharomyces cerevisiae* • fungus • *Candida albicans* • red blood cell • drug delivery • density gradient ultracentrifugation • electron microscopy

Reconstituted high density lipoproteins (rHDLs) have been studied extensively, and in certain instances, these macromolecular assemblies of lipid and protein have been used for beneficial purposes. Recently, rHDL infusion has shown therapeutic promise for regression of atherosclerotic lesions (1). In other cases, hydrophobic biomolecules, including anticancer (2, 3) and antiviral (4) drugs, have been incorporated into rHDL. In this study, we hypothesized that the macrolide polyene antibiotic amphotericin B (AMB) (**Fig. 1**) could be incorporated into rHDL with retention of its antifungal properties. AMB, which is widely used in the treatment of systemic fungal infections, binds preferentially to 24 substituted sterols, such as the fungal cell membrane component ergosterol (5, 6). AMB-ergosterol complexes form pores in the fungal membrane that cause leakage of cellular contents and cell death (7, 8). Specificity is achieved by the fact that fungal membranes contain ergosterol, whereas mammalian host membranes possess cholesterol. Toxicity to host tissues occurs, however, because AMB's binding preference for ergosterol over cholesterol is not absolute.

A number of strategies have been used to address issues related to the poor water solubility and toxicity of AMB. The bile salt deoxycholate has been used to generate AMB-deoxycholate mixed micelles. Although effective at solubilizing AMB, this preparation manifests significant in vivo toxicity at efficacious doses. More recently, it was shown that AMB toxicity toward mammalian tissues can be reduced significantly by complexing the antibiotic with lipids. Lipid formulations include a colloidal dispersion of AMB and cholesteryl sulfate (9), various liposomal preparations (10), and a suspension of dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), and AMB that exist as ribbon-like structures with a diameter of 1.6–11.1 μm (11).

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Abbreviations: AMB, amphotericin B; apoA-I, apolipoprotein A-I; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; IC_{90}, concentration at which 90% growth inhibition occurs; RBC, red blood cell; rHDL, reconstituted high density lipoprotein; YEPD, yeast extract peptone glucose broth medium.

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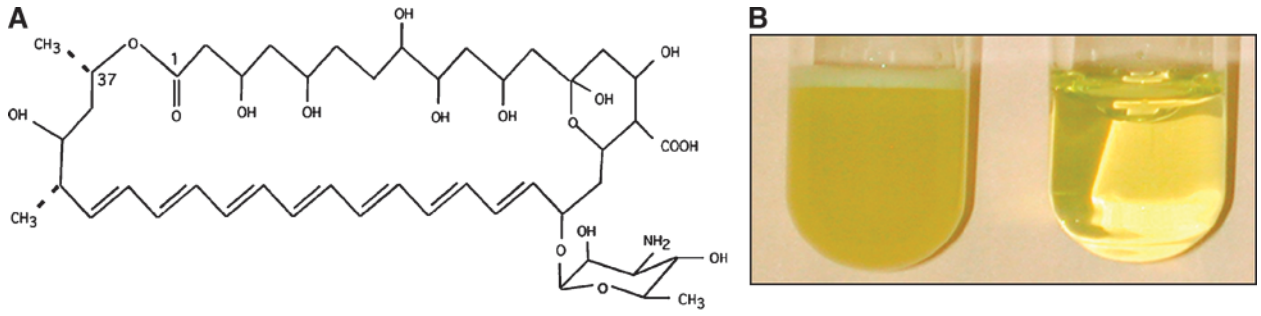

Fig. 1. Apolipoprotein A-I (apoA-I)-induced solubilization of amphotericin B (AMB). A: AMB structure. B: Phospholipid vesicle/AMB mixture before (left) and after (right) the addition of apoA-I.

A well-known property of human apolipoprotein A-I (apoA-I) is its ability to solubilize vesicles of DMPC (12, 13) or DMPG (14), transforming them into nanometer-scale, discoidal rHDLs. Characterization studies have provided evidence that the particles generated exist as a phospholipid bilayer whose periphery is circumscribed by apolipoprotein molecules (15). Detailed examination of the organization and alignment of the protein component of these complexes has shown that apolipoprotein α-helix segments circumscribe the periphery of the bilayer disk, with the long axis of their helical segments aligned perpendicular to the phospholipid fatty acyl chains (16–18). The present results show that inclusion of up to 20% (w/w) AMB into a suitable phospholipid vesicle substrate has no apparent effect on apoA-I-induced vesicle transformation into rHDLs and that the product particles harboring AMB are biologically active.

MATERIALS AND METHODS

Materials

AMB (USP grade) was obtained from Research Organics, Inc. DMPC and DMPG were from Avanti Polar Lipids, Inc. Recombinant apoA-I was produced as described previously (19). Liposomal AMB (AmBisome) and AMB-deoxycholate (Fungizone) were resuspended in sterile deionized water according to the manufacturers' instructions.

AMB-rHDL preparation

Seven milligrams of DMPC and 3 mg of DMPG were dissolved in chloroform-methanol (3:1, v/v) and dried under a stream of nitrogen gas, coating the vessel wall with the phospholipid. The tube was then lyophilized for a minimum of 2 h to remove residual organic solvent. After this, the lipids were dispersed in 1 ml of PBS (20 mM sodium phosphate, pH 7.0, and 150 mM sodium chloride) by vortexing. To the dispersed lipid, 2.5 mg of AMB from a stock solution (30 mg/ml in DMSO) was added. Subsequently, 4 mg of apoA-I in 2.0 ml of PBS was added, and the solution (3.1 ml final volume) was incubated at 24°C. The addition of apoA-I in buffer leads to a time-dependent decrease in sample turbidity, consistent with the formation of rHDLs. Full sample clarity is achieved by mild bath sonication (30 s to several minutes). The solution was dialyzed overnight against PBS to remove DMSO and filter-sterilized before use.

Density gradient ultracentrifugation

A solution of AMB-rHDL was adjusted to a density of 1.33 g/ml by the addition of solid KBr in a final volume of 5.5 ml. The sample was transferred to a 13 ml centrifuge tube, overlayered with saline, and centrifuged at 50,000 rpm for 26 h in a Ti50 rotor. After centrifugation, the tube contents were fractionated (0.5 ml), and the fractions containing AMB-rHDL were pooled and dialyzed.

Analytical procedures

Protein concentrations were determined by the bicinchoninic acid assay (Pierce Chemical Co.) with BSA as a standard. Choline-containing phospholipids were quantified by an enzyme-based colorimetric assay (Wako). Nondenaturing PAGE was performed on 4–20% acrylamide slab gels. Samples were electrophoresed at 150 V constant voltage for 20 h. Gels were stained with Coomassie blue, and the relative mobility of AMB-rHDL complexes was compared with those of standards of known size, including BSA, lactate dehydrogenase, catalase, horse ferritin, and thyroglobulin.

Electron microscopy

rHDL samples were prepared for negative staining electron microscopy using a modification of the method described by Valentine, Shapiro, and Stadtman (20). A small piece of carbon-coated mica (~3 × 8 mm) was inserted into a solution of AMB-rHDL (~200 μg protein/ml in PBS, pH 7.4). The carbon-coated mica was inserted at a shallow angle, so that part of the carbon film detached from the mica and floated onto the rHDL solution. After 1 min, the carbon film was retracted from the solution, floated for 1 min on deionized water, and transferred to a 2% solution of phosphotungstic acid, pH 7.4. Subsequently, the carbon film was lifted from the phosphotungstic acid solution onto a 300 mesh copper grid, allowed to air dry, and analyzed with a JEOL 1230 transmission electron microscope at an acceleration voltage of 100 kV.

Ultraviolet/visible absorbance spectroscopy

Absorbance spectroscopy was performed on a Perkin-Elmer Lambda 20 spectrometer. AMB levels were determined using an extinction coefficient at 416 nm = 1.214×10^5 M^{-1} cm^{-1} in DMSO (21). Samples were scanned from 300 to 450 nm. Scans of rHDL samples in aqueous media were obtained in PBS. Scans of AMB alone in PBS were obtained after DMSO was removed under a stream of nitrogen and lyophilization, addition of buffer to the dried antibiotic, and dispersion of AMB by bath sonication. Under these conditions, the AMB in the sample appears as a colloid suspension rather than a solution of AMB.

Yeast growth inhibition assays

Cultures of the yeast *Saccharomyces cerevisiae* were grown in yeast extract peptone glucose broth medium (YEPD; Teknova, Hollister, CA). Twenty microliters of a saturated overnight culture was used to inoculate 5 ml of YEPD in the absence or presence of the indicated amounts of a given AMB formulation. Cultures were grown for 16 h at 30°C with rotation, and the extent of culture growth was monitored by measuring sample turbidity at 600 nm.

Pathogenic fungi growth inhibition assays

Microtiter broth growth inhibition assays were conducted with three species of pathogenic fungi: *Candida albicans* [American Type Culture Collection (ATCC) strain 90028], *Aspergillus fumigatus* (ATCC strain 16424), and *Cryptococcus neoformans* (isolate H99, ATCC strain 208821). Fungi were cultured in RPMI 1640 medium buffered with MOPS to pH 7.0. The final inoculum was 1×10^6 cells/ml. Experiments were performed in triplicate at 37°C for 48 h according to established protocols (22, 23). All samples tested were soluble in the standard RPMI medium used, and no precipitation or interference was seen in any of the samples tested against either fungal species. Inhibitory activity was determined from cultures grown with varying amounts of a given AMB formulation ranging from 0.01 to 16 μg/ml.

Erythrocyte hemolysis assay

Whole blood collected from healthy volunteers was centrifuged for 3 min at 1,000 *g*. The plasma fraction was removed, and the red blood cells (RBCs) were diluted 1:10 in PBS (or 150 mM NaCl, pH 7–7.4). Deionized water was used as a 100% lysis control. Aliquots (25 μl) of diluted RBCs were transferred to 1.5 ml microfuge tubes, and a given amount of AMB formulation or buffer was added to a final volume of 500 μl. Reaction tubes were incubated at 37°C for 1 or 20 h and centrifuged at 1,000 *g* for 4 min. An aliquot (200 μl) of the supernatant was added to wells of a microtiter plate containing 25 μl of Drabkins reagent, and sample absorbance at 540 nm was measured on a Spectramax 340 plate reader.

Cell culture studies

HepG2 (human hepatoma) cells were grown in MEM (Gibco) supplemented with 4 mM L-glutamine, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. Cells were split twice weekly into fresh medium and maintained at 37°C in a humidified 5% CO_2 atmosphere. For experiments, the cells were seeded on 24-well plates with 2 ml of complete medium, 2×10^4 cells per well. The medium was aspirated after 48 h, the cells were washed with MEM containing 4% FBS, and the wells were each given 2 ml of fresh MEM with 4% FBS. Treatment of cells with AMB commenced 48 h after seeding at concentrations ranging from 0 to 25 μg/ml. Assays were performed in triplicate. After 20 h of culturing in the presence or absence of antibiotic, cell viability was measured spectrophotometrically using the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assay as described by the manufacturer (Sigma). Values are expressed as percentages of absorbance of untreated control cells.

In vivo toxicity studies

Female BALB/c mice (6–8 weeks old) were divided into groups of three mice each and administered 1, 2, 5, 10, and 15 mg/kg AMB-rHDL intraperitoneally (0.1 ml total volume). At 2 and 6 h after AMB-rHDL injection, and every 24 h thereafter for 7 days, mice were observed for weight loss or abnormalities in appearance and behavior. Blood was drawn 24 h after drug administration, and liver (alanine aminotransferase and aspartate aminotransferase) and kidney (urea and creatinine) function markers were analyzed.

Efficacy studies

Female BALB/c mice (6–8 weeks old) were divided into groups of 10 mice each for a dose-response study of AMB-rHDL efficacy. Each group was inoculated with 5×10^5 blastospores of *Candida albicans* (ATCC strain 90028). Two hours after inoculation, mice were treated with PBS or 0.25, 0.5, 1.0, 2.5, or 5 mg/kg AMB-rHDL. Treatment was administered once a day for 5 days. Over the course of the 28 day observation period, mice were examined twice daily for mortality, weight loss, failure to take food or water, and abnormalities in appearance and behavior. In vivo toxicity and efficacy studies were conducted at the Center for Medical Mycology at Case Western Reserve University.

RESULTS AND DISCUSSION

AMB-rHDL formation and characterization

Addition of apoA-I to an aqueous dispersion of DMPC/DMPG/AMB (7:3:2.5, w/w) induced clarification of the reaction mixture with complete solubilization of the antibiotic (Fig. 1). Whereas the substrate phospholipid-AMB mixture is opaque and pellets upon centrifugation at 13,000 *g*, the reaction product is a transparent yellow solution that is unaffected by centrifugation. From these results, it is evident that apoA-I has interacted with the lipid mixture to form soluble, antibiotic-bearing lipid complexes. Dialysis of the sample against PBS results in the loss of <10% of the original AMB. Upon increasing the amount of AMB present in the reaction mixture, rHDL formation was incomplete and significant amounts of AMB precipitated. Likewise, using the classical cholate dialysis method for the preparation of rHDL (24), although AMB was solubilized in the reaction mixture, far less antibiotic was incorporated into the product palmitoyloleoylphosphatylcholine rHDL, because of the loss of AMB during the dialysis step required to remove the detergent. This result suggests that AMB-rHDL formation may be limited to phospholipids that apoA-I is able to transform into rHDL in the absence of detergent treatment/dialysis. To date, we have found efficient AMB-rHDL formation with DMPC, DMPG, and egg sphingomyelin. In trial experiments, in addition to the 7:3 ratio described above, AMB-rHDL can be prepared using 100% of any one of these phospholipid substrates.

To characterize the nature of the particles formed, the sample was subjected to density gradient ultracentrifugation. After centrifugation, the tube contents were fractionated and analyzed for protein, phospholipid, AMB, and density. As shown in **Fig. 2**, although some material was recovered at the top of the centrifuge tube (d = 1.136 g/ml), the majority of the AMB was recovered in a distinct peak that comigrates with protein and phospholipid at a density of 1.16 g/ml. The rHDL recovered in the 1.16 g/ml fraction was present in a DMPC/apoA-I/AMB ratio of 1.5:1.0:0.6 (w/w). Assuming maintenance of a

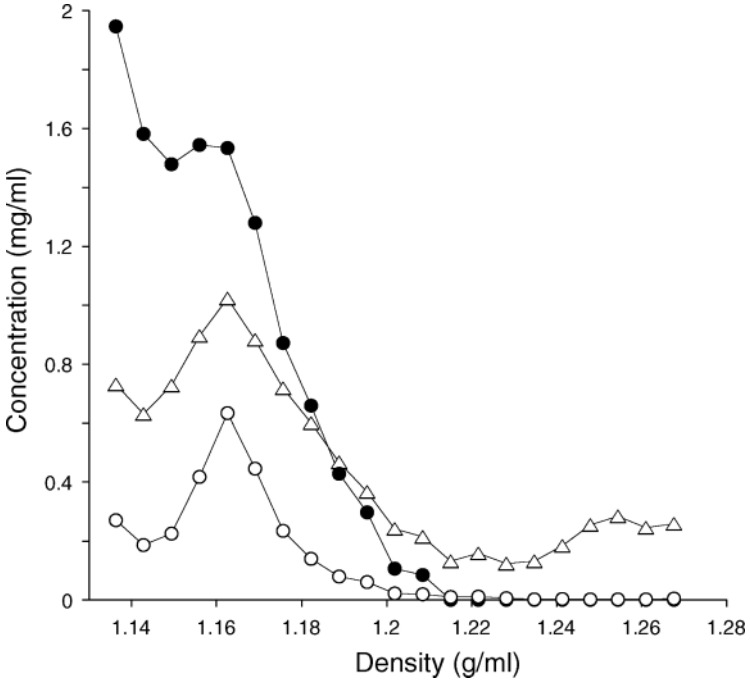

Fig. 2. Density gradient ultracentrifugation of apolipoprotein-phospholipid-AMB complexes. A preparation of AMB-reconstituted high density lipoprotein (rHDL) was adjusted to a density of 1.33 g/ml by the addition of solid KBr in a final volume of 5.5 ml. This solution was transferred to a 13 ml centrifuge tube, overlayered with saline, and centrifuged at 50,000 rpm for 26 h in a Ti50 rotor. After centrifugation, the tube contents were fractionated into 0.5 ml fractions, and the density and protein, AMB, and phospholipid contents in each fraction were determined. Open triangles, protein; closed circles, phospholipid; open circles, AMB.

7:3 DMPC/DMPG weight ratio in the product rHDL, a phospholipid/apoA-I/AMB weight ratio of 2.1:1.0:0.6 may be estimated, corresponding to a 100:1:20 molar ratio.

Negative stain electron microscopy revealed the presence of disk-shaped complexes that display a tendency to stack (**Fig. 3A**). Stacked disks are not thought to exist in solution but to occur as a result of the dehydration procedure used in the sample preparation for electron microscopy (24). The majority of the particles have diameters in the range of 8–10 nm. The presence of a few larger particles may be attributable to particle fusion or aggregation. The particle size distribution of AMB-rHDL was further evaluated by native PAGE (Fig. 3B). From comparison with the relative migration of known standards, the Stokes diameter of AMB-rHDL is ~8.5 nm. Freezing and thawing or lyophilization and redissolving in buffer did not affect sample appearance or electrophoretic mobility.

Spectroscopic analysis of AMB-rHDL

The absorbance spectrum of AMB in DMSO is characteristic of monomeric AMB (**Fig. 4**), with distinct maxima at 416, 392, and 372 nm. By contrast, absorbance spectra of an aqueous dispersion of AMB in PBS lacked these extrema. The spectrum of AMB-rHDL in PBS revealed spectral maxima at 413, 385, 362, and 345 nm. The increase in absorbance at 413 and 385 nm for AMB-rHDL versus the aqueous AMB dispersion is consistent with the insertion of AMB into a membrane environment (25, 26). Furthermore, the prominent spectral maximum at 345 nm in these samples indicates the presence of self-associated or aggregated AMB (27). A corresponding spectrum of Fungizone [AMB/sodium deoxycholate (5:4, w/w)] in PBS was similar to that reported by Barwicz, Gruszecki, and Gruda (28) and displayed a prominent spectral maxima at 329 nm with smaller absorbance peaks at longer wavelengths, consistent with AMB aggregation (data not shown). An interesting feature of the present AMB-rHDL preparation is the complete absence of solution turbidity, a property that allows for the characterization of AMB spectral properties without the light-scattering artifacts often associated with liposomes or vesicles. As such, AMB-rHDL may provide a system for investigation of the molecular organization of AMB and phospholipid in these complexes, which, at present, is not known.

Biological activity of AMB-rHDL

To evaluate whether the incorporation of AMB into rHDL affects its biological activity, growth inhibition assays

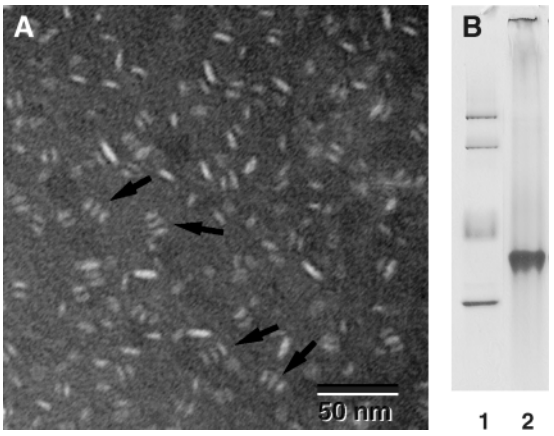

Fig. 3. Characterization of AMB-rHDL. A: Electron microscopy. A sample of AMB-rHDL was stained with 2% phosphotungstic acid and analyzed with a JEOL 1230 transmission electron microscope at an acceleration voltage of 100 kV. Arrows indicate short stacks of AMB-rHDL particles. Magnification = 288,000×. B: Native gradient polyacrylamide gel electrophoresis. Lane 1, molecular weight standards; lane 2, AMB-rHDL.

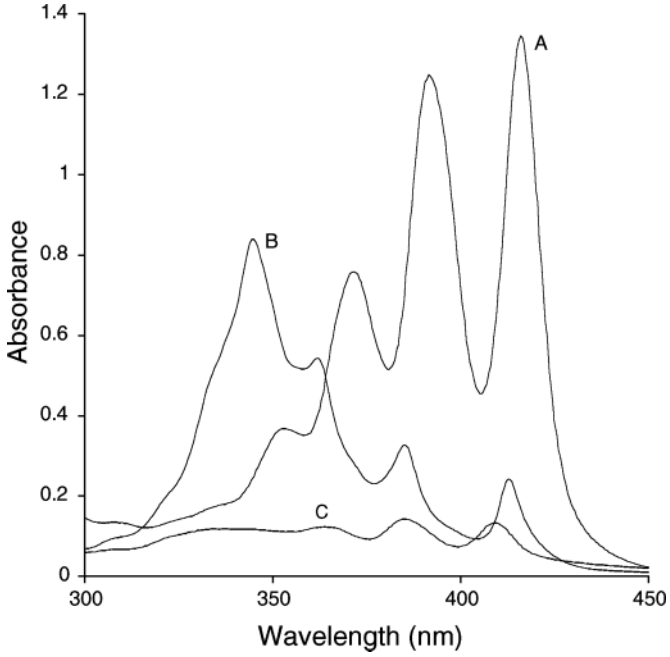

Fig. 4. Ultraviolet/visible absorbance spectra of AMB. Samples of AMB (12 μg/ml) in DMSO (trace A), rHDL (trace B), or PBS (trace C) were scanned from 300 to 450 nm.

were performed with the yeast *S. cerevisiae*. Control rHDLs lacking AMB had no effect on yeast growth. By contrast, the data in **Fig. 5** show that AMB-rHDL effectively inhibited *S. cerevisiae* growth, with inhibition observed at a lower AMB concentration than either AMB-deoxycholate

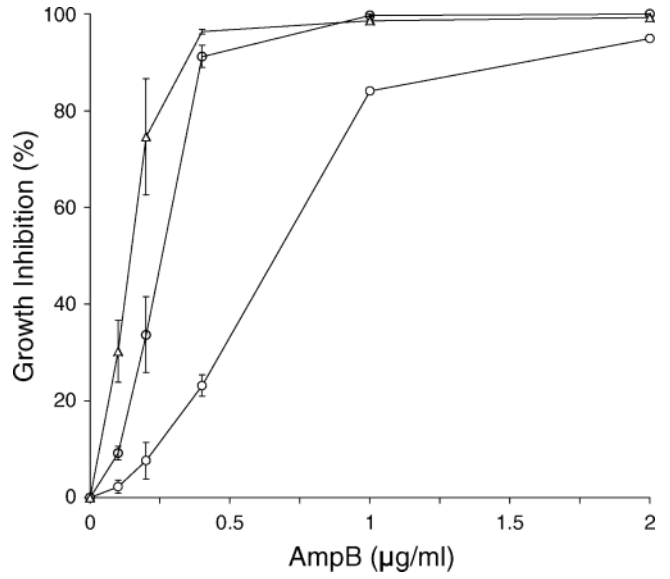

Fig. 5. Effect of AMB-rHDL, liposomal AMB, and AMB-deoxycholate on yeast growth. Cultures of *S. cerevisiae* were grown in yeast extract peptone glucose broth medium in the presence of the indicated amounts of different AMB formulations. The cultures were grown for 16 h at 30°C, and the extent of culture growth was determined spectrophotometrically. Open triangles, AMB-rHDL; closed circles, AMB-deoxycholate; open circles, liposomal AMB. Values reported are means ± SD (n = 3). When not shown, the error bar is smaller than the symbol.

micelles or liposomal AMB. Whereas 50% growth inhibition was achieved with AMB-rHDL at 0.14 μg/ml, this level of growth inhibition required 0.25 μg/ml AMB-deoxycholate and 0.65 μg/ml liposomal AMB.

Pathogenic fungi growth inhibition assays

Given the apparent strong in vitro growth inhibition activity of AMB-rHDLs toward yeast, their ability to inhibit the growth of disease-causing pathogenic fungi was examined in microtiter broth assays. Each of the fungal species tested causes serious illness in humans and represents an ever-increasing infection risk in immunocompromised individuals. As shown in **Table 1**, AMB-rHDL was effective against *C. albicans*, *A. fumigatus*, and *C. neoformans* at a lower concentration than liposomal AMB. Control rHDL lacking AMB did not inhibit fungal growth.

RBC lysis

To characterize toxicity toward cholesterol-containing membranes, various AMB formulations were evaluated in terms of their ability to induce hemolysis of human RBCs. In control incubations, no hemolysis was observed upon incubation of erythrocytes in PBS, whereas 100% hemolysis occurred in deionized water. After a 1 h incubation, AMB-deoxycholate induced hemolysis at relatively low concentrations, whereas liposomal AMB and AMB-rHDL required much higher concentrations to induce hemolysis (**Fig. 6**). Although some hemolysis occurred at longer incubation times (20 h) with AMB-rHDL, the observed hemolysis was still lower than that seen for AMB-deoxycholate at 1 h (data not shown).

Cell culture studies

The extent to which AMB toxicity toward nucleated cells in culture is affected by incorporation into rHDL was examined using HepG2 cells (**Fig. 7**). In the case of AMB-deoxycholate, cell damage/death occurred at low concentrations of AMB, with nearly 100% cell death at 25 μg/ml. By contrast, similar concentrations of AMB presented as rHDL induced <5% decrease in cell viability.

In vivo toxicity

To evaluate the acute toxicity of AMB-rHDL, immunocompetent mice were administered a single intraperito-

TABLE 1. Effect of AMB formulation on pathogenic fungal growth

	IC_{90}		
Fungal Species	AMB-Reconstituted High Density Lipoprotein	Liposomal AMB	AMB-Deoxycholate
		μg/ml	
C. albicans	0.03	0.24	0.05
A. fumigatus	0.10	0.47	0.42
C. neoformans	0.05	0.24	0.05

AMB, amphotericin B; IC_{90}, concentration at which 90% growth inhibition occurs. Assays were performed in triplicate, and in each case, IC_{90} values were identical. Fungi were cultured in RPMI medium buffered with MOPS to pH 7.0. The final inoculum was 1×10^6 cells/ml. Experiments were performed at 37°C for 48 h.

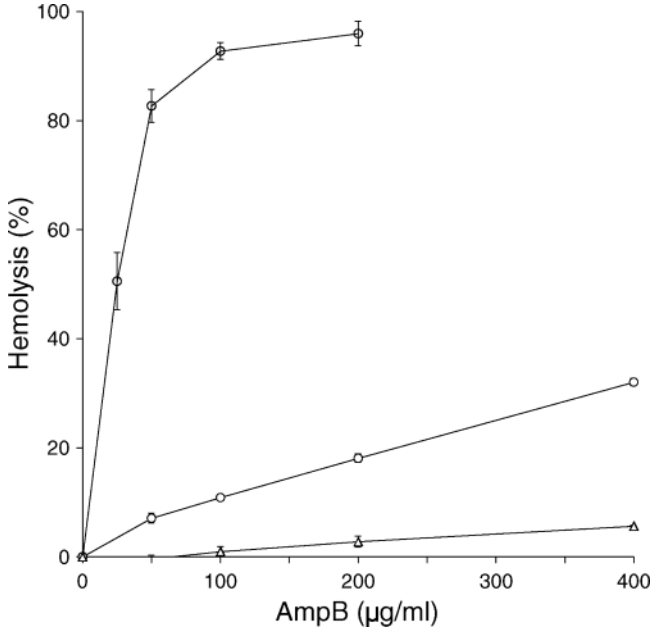

Fig. 6. Effect of AMB on red blood cell hemolysis. Fresh human erythrocytes were incubated in the presence of the indicated amounts of AMB-deoxycholate, liposomal AMB, or AMB-rHDL. Reaction tubes were incubated at 37°C for 1 h and centrifuged at 1,000 *g* for 4 min. An aliquot (200 μl) of the supernatant was added to wells of a microtiter plate containing 25 μl of Drabkins reagent, and sample absorbance at 540 nm was measured on a Spectramax 340 plate reader. Open triangles, AMB-rHDL; closed circles, AMB-deoxycholate; open circles, liposomal AMB. Values reported are means ± SD (n = 3). When not shown, the error bar is smaller than the symbol.

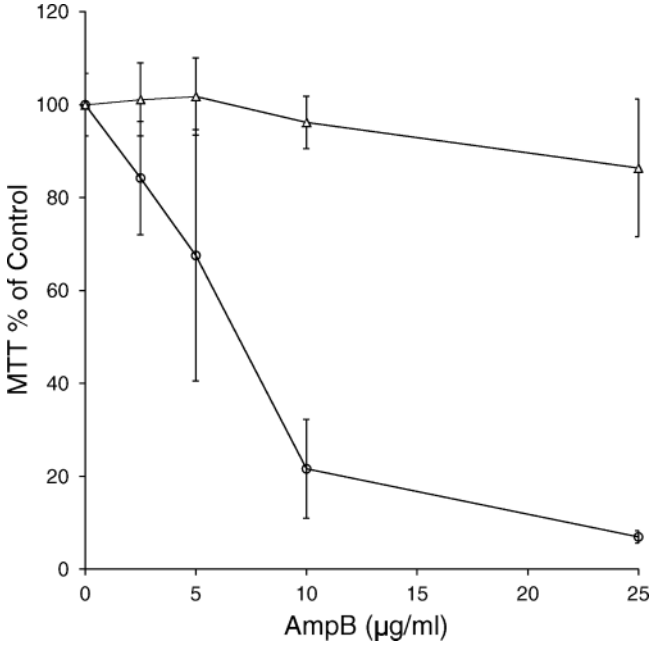

Fig. 7. Effect of AMB on the viability of cultured HepG2 cells. Cultures of HepG2 cells were treated with AMB at concentrations ranging from 0 to 25 μg/ml. After 20 h of further culturing in the presence or absence of antibiotic, cell viability was measured using the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assay. Percentage cell viability was derived from the difference in absorbance (570 and 630 nm) at each AMB concentration. Open triangles, AMB-rHDL; closed circles, AMB-deoxycholate. Values reported are means ± SD of three determinations.

neal injection of AMB-rHDL (1, 2, 5, 10, or 15 mg/kg). Acute toxic effects were observed in the 15 mg/kg AMB-rHDL group. Two of the three mice given this dose did not survive the treatment. For treatment regimens of 5 mg/kg or less, nominal weight loss occurred. In mice treated with 10 mg/kg AMB-rHDL, significant weight loss was observed on day 2, with subsequent recovery of up to 90% of body weight by day 7. At AMB concentrations up to 10 mg/kg AMB-rHDL, markers of nephrotoxicity (0.16 mg/dl creatinine versus 0.10 mg/dl in control mice; there was no change in urea) and hepatotoxicity remained below toxic thresholds. In the latter case, aspartate aminotransferase activity was 125 U/l in control mice versus 150 U/l in mice administered 10 mg/kg AMB-rHDL. Likewise, alanine aminotransferase activity was 45 U/l in both control mice and mice administered 10 mg/kg AMB-rHDL.

AMB-rHDL efficacy in the treatment of systemic candidiasis

To evaluate the efficacy of AMB-rHDL in the treatment of systemic fungal disease, mice were infected with *C. albicans* followed by treatment (daily for 5 days after infection) with AMB-rHDL (**Fig. 8**). All uninfected mice treated with PBS survived the 28 day period of observation, whereas 8 of 10 infected mice administered PBS alone did not survive beyond day 6. Those infected mice treated with AMB-rHDL, however, showed a greatly improved survival pattern. With the exception of a single dead mouse on day 5 in the 5 mg/kg group (data not shown), all remaining mice, plus all mice in the 2.5 and 1.0 mg/kg treatment groups, survived the entire observation period. At 0.25 and 0.5 mg/kg, some mortality occurred after day 15. By the end of the 28 day observation period, five mice in the 0.5 mg/kg group and six mice in the 0.25 mg/kg group survived.

Collectively, the results presented here suggest that AMB-rHDL is a novel formulation of AMB that overcomes the poor solubility of this drug with retention of potent antifungal activity. Compared with other lipid formulations of AMB, rHDLs possess unique properties that may be exploited in creating a second generation or designer rHDLs to achieve enhanced efficacy. In terms of bioavailability, it is known that commercial liposomal AMB was engineered to possess slow AMB release kinetics (29). This property, which is not shared by AMB-rHDL, may explain the effectiveness of AMB-rHDL in in vitro pathogenic fungi growth inhibition assays. It is conceivable that the nanometer-scale size and particle geometry of AMB-rHDL may contribute to AMB bioavailability. For example, because both faces of an rHDL disk are accessible to the external environment, AMB present in either leaflet of the bilayer is accessible to target membranes. By contrast, one-half of liposomal AMB molecules would be expected to orient toward the interior of the liposome, a location where it would be inaccessible to potential membrane

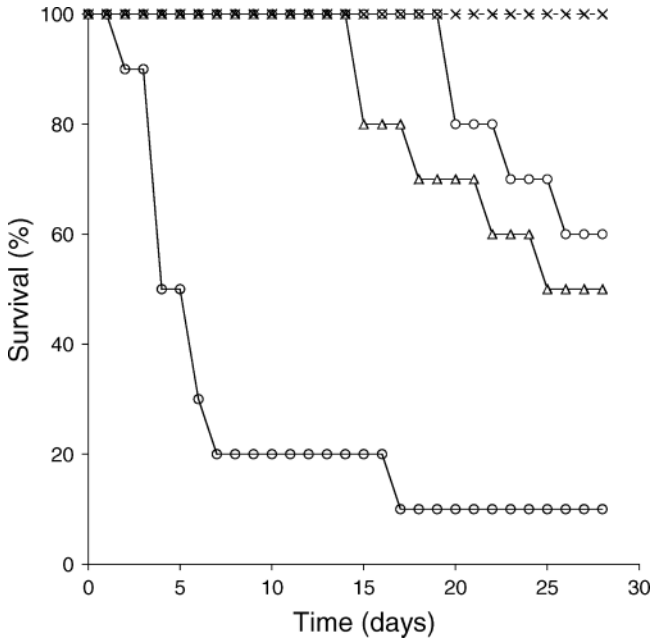

Fig. 8. Effect of AMB-rHDL on the survival of *C. albicans* infected mice. Female BALB/c mice were inoculated with 5×10^5 blastospores of *C. albicans*. Two hours after inoculation and once per day for 5 consecutive days, mice were treated with PBS (closed circles) or 0.25 (open circles), 0.5 (open triangles), or 1.0 or 2.5 (crosses) mg/kg AMB-rHDL. Survival was monitored over the course of a 28 day observation period.

interaction sites. Also, AMB-rHDLs are far smaller than liposomal AMB and, hence, may be able to access fungal cell surface locations that liposomes or other AMB lipid formulations cannot. A liposome with a diameter in the range of 60 nm has a surface area of ~11,309 nm^2, whereas an rHDL particle with a diameter of 10 nm will have a surface area (of one face of the discoidal bilayer) of ~78 nm^2. Thus, the surface material in one liposomal AMB particle would give rise to >140 rHDL particles.

Another consideration is the presence of protein as an intrinsic component of AMB-rHDL. It has been suggested that members of the exchangeable apolipoprotein family interact with the β-1,3-glucan component of fungal cell walls (30). It is conceivable that the protein component of AMB-rHDL could facilitate interaction of the rHDL with the fungal cell wall, thereby increasing AMB contact with target membranes. By the same token, the presence of protein as a component of rHDL imparts versatility. For example, substitution of apoE for apoA-I would be expected to confer recognition by members of the low density lipoprotein receptor family, potentially targeting rHDL for internalization by certain cell types (31). On the other hand, protein engineering may be used to generate modified apolipoproteins that retain phospholipid solubilization properties yet are conferred with additional features, such as receptor binding specificity (32). Given that cytotoxicity of free or liposomal AMB is attenuated by the interaction of HDL in plasma (33), AMB-rHDL may offer advantages as a delivery vehicle for this poorly soluble yet potent antifungal agent.

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In the article "Reconstituted high-density lipoprotein enriched with the polyene antibiotic, amphotericin B" by Michael N. Oda et al., published in the February 2006 issue of the *Journal of Lipid Research* (Volume 47, pages 260–267), due to a production error Figures 5, 6, 7 and 8 contain symbols that should be closed circles but, instead, appear as open circles. Corrected figures are displayed below.

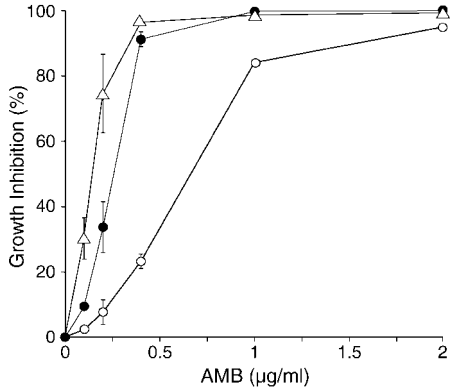


Fig. 5. Effect of AMB-rHDL, liposomal AMB, and AMB-deoxycholate on yeast growth. Cultures of *S. cerevisiae* were grown in yeast extract peptone glucose broth medium in the presence of the indicated amounts of different AMB formulations. The cultures were grown for 16 h at 30°C, and the extent of culture growth was determined spectrophotometrically. Open triangles, AMB-rHDL; closed circles, AMB-deoxycholate; open circles, liposomal AMB. Values reported are means ± SD (n = 3). When not shown, the error bar is smaller than the symbol.

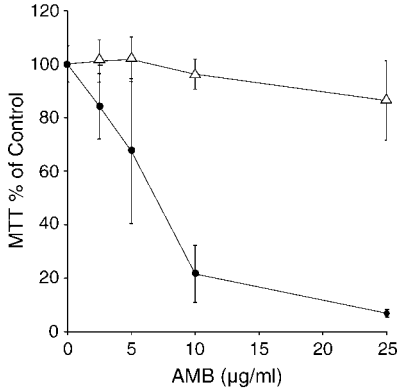


Fig. 7. Effect of AMB on the viability of cultured HepG2 cells. Cultures of HepG2 cells were treated with AMB at concentrations ranging from 0 to 25 μg/ml. After 20 h of further culturing in the presence or absence of antibiotic, cell viability was measured using the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assay. Percentage cell viability was derived from the difference in absorbance (570 and 630 nm) at each AMB concentration. Open triangles, AMB-rHDL; closed circles, AMB-deoxycholate. Values reported are means ± SD of three determinations.

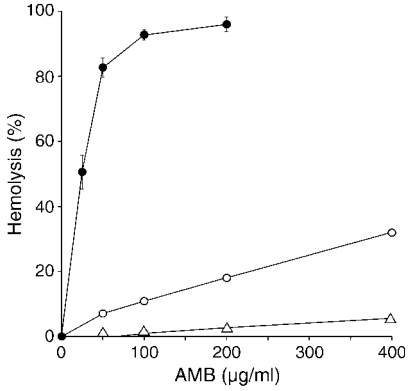
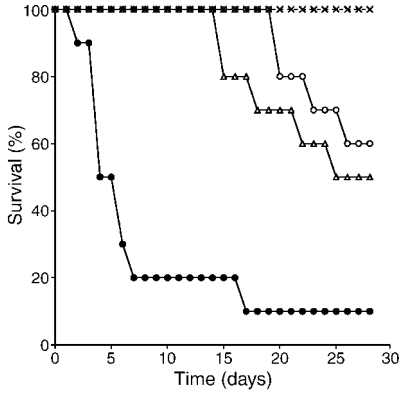


Fig. 6. Effect of AMB on red blood cell hemolysis. Fresh human erythrocytes were incubated in the presence of the indicated amounts of AMB-deoxycholate, liposomal AMB, or AMB-rHDL. Reaction tubes were incubated at 37°C for 1 h and centrifuged at 1,000 *g* for 4 min. An aliquot (200 μl) of the supernatant was added to wells of a microtiter plate containing 25 μl of Drabkins reagent, and sample absorbance at 540 nm was measured on a Spectramax 340 plate reader. Open triangles, AMB-rHDL; closed circles, AMB-deoxycholate; open circles, liposomal AMB. Values reported are means ± SD (n = 3). When not shown, the error bar is smaller than the symbol.



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